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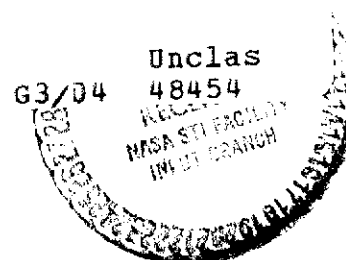
## Semi-Annual Status Report

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## ABSTRACT

During the six months covered by this report (November 1973 - 30 April 1974), the Laboratory of Aerospace Biology conducted an experiment to determine the effects of air velocity on metabolic performance. The significance of this research relates to the use of high forced airflow in a closed environment as a mechanism to control the concentration of volatile animal wastes. Air velocities between 100 and 200 ft/min are without significant effects on the metabolism of rats. At velocities of 200 ft/min and above, oxygen consumption and CO<sub>2</sub> production as well as food consumption increase. In most instances, the changes are on the order of 5-10%. At the same time, the RQ for the animals increased slightly and generally correlated well with the oxygen consumption and CO<sub>2</sub> production.

The Laboratory of Aerospace Biology also conducted its concluding experiments on the nature of inert gas narcosis during this six month period. Utilizing the electron transport particle prepared from rat liver mitochondria, the NADH:O<sub>2</sub> oxidoreductase activity was inhibited in vitro by argon gases but only at relatively high pressures (120 ATM but not at 50 or 75 ATM). Utilizing the luciferin-luciferase enzyme system prepared from firefly tails, both argon and nitrous oxide showed marked inhibition. Luciferase was inhibited by argon at pressures of 50 and 100 ATM and the ED<sub>50</sub> for N<sub>2</sub>O was 23 ATM. In these in vitro

experiments, both halothane and methoxyflurane were observed to be rather potent inhibitors of the  $\text{NADH}:\text{O}_2$  oxidoreductase system. These experiments suggest that the mechanism of inert gas narcosis is not mandatorily related to a membrane surface phenomenon.

The report also includes new data and an up-date of a technique developed in this laboratory for a rapid quantitative measure of comparative metabolic activity which has been prepared in manuscript form for submission to the editors of Comparative Biochemistry and Physiology.

# EFFECTS OF CHRONIC EXPOSURE TO AIRFLOW OF MODERATE VELOCITIES ON METABOLISM

## Introduction:

The control of volatile animal wastes under the weightless environment of space is a problem of no small magnitude. Although several methods have been devised to control and contain volatile waste material, the one which currently shows some promise involves the removal of this material by forced airflow; a similar method was used to control volatile human waste on Skylab. Even though the velocities of airflow under consideration are not great, they may be sufficient to pose a chronic environmental stress on the animals subjected.

At the request of Dr. Emily Holton, NASA-Ames Research Center, an investigation was conducted on the effects on the metabolism of white rats chronically exposed to airflow at several velocities under 1 ATA air at an environmental temperature within the animals' thermal neutral range. The results of this investigation indicate that air velocities in excess of 200 feet/minute do impose a stress on the animals. Although the changes observed are fairly small, they may prove to be significant.

## Method and Materials:

To conduct this study a metabolic chamber was fitted with tubular cage to house the animals, fan and other equipment. The cage was constructed of 26 gauge stainless steel and measured 31" long by 12" in diameter. The bottom of this tube was sealed except for a rectangular

hole for the fan discharge and two 2.5" x 8" slots cut in the tube wall for regulating airflow. The fan, a Dayton model 4C264 centrifugal blower with a no-load rating of 465 ft<sup>3</sup>/min at sea level, was mounted directly over the discharge hole. The floor of the animal compartment was set on "L" brackets 10" below the upper end of the tube and was constructed of heavy-gauge expanded stainless steel having an open cross section of 0.8 ft<sup>2</sup>. A circular drop tray was similarly mounted on brackets midway between the cage floor and the fan. Its diameter was 1" less than the diameter of the tube permitting a relatively unrestricted flow of air from the animal compartment to the fan. Additionally, a 3.5" in diameter hole was cut in the center of the tray to insure a uniform cross section of airflow. This hole was surrounded by 1.5" high collar and covered with a 4" in diameter cone to retard the deposition of animal waste in the fan compartment. The upper end of the tube was covered with an open-cell material (Hexcel) 1.5" thick to reduce air turbulence within the animal compartment.

Mounted in the animal compartment were a food hopper, water cup, 4-watt fluorescent lamp, thermoswitch and thermocouple. The food hopper was positioned out of the air stream along the wall of the animal compartment and held about 425 gm of Purina Rat Chow. The water cup was mounted next to the food hopper and had a 75 ml capacity; it was automatically filled every 2 hours. The fluorescent lamp was secured above the food hopper and water cup at the edge of the air stream

and was controlled by a time switch providing a 12:12 LD cycle. The thermoswitch and thermocouple were mounted together in a wire mesh enclosed pod located in the air stream 8" above the animal compartment floor on the central axis of the tube.

When the chamber was sealed, fresh air was continuously introduced at the top of the chamber at the rate of 7.4 l/min, pulled through the animal compartment by the blower, and exhausted at the bottom of the chamber. The average O<sub>2</sub> and CO<sub>2</sub> concentrations within the chamber were about 20% and 0.6%, respectively.

Oxygen consumption and CO<sub>2</sub> production were derived from measurements made at 4 hour intervals 5 times daily starting at 0800 hours. Oxygen and CO<sub>2</sub> in the effluent line of the chamber were quantified by gas chromatography using a Varian A350B gas chromatograph equipped with a dual thermal conductivity detector. CO<sub>2</sub> was resolved on a 0.25" x 24" silical gel column operated at 120°C while oxygen and nitrogen were resolved on a 0.25" x 120" mole sieve 5 A column operated at room temperature. Helium was used as the carrier gas. Gas samples were injected onto the columns at a pressure of 1 ATA with a sample valve equipped with a 0.5 ml sample loop. The gas standard containing 1.56% CO<sub>2</sub>, 20.7% O<sub>2</sub> and 77.7% nitrogen was applied to the columns at the same pressure and volume. The CO<sub>2</sub> and O<sub>2</sub> concentrations were calculated by linear extrapolation of sample peak areas based on areas of the gas standard peaks. Peak areas were calculated by multiplying peak width at half height by peak height.

The overall error of this method averaged  $\pm 5\%$ . Sample data were converted to l/day based on the gas flow through the chamber over a 24-hour period beginning at midnight.

Air velocity was periodically monitored with a Thermo-Systems model 4100 ionization-type airflow meter. The air stream was measured at 5 equidistant points midway between the center and periphery of the animal compartment floor and averaged. The average value was corrected to 1 ATA and  $28^{\circ}\text{C}$ .

Four male rats (Caworth Farms) weighing  $300 \pm 20$  gms were placed in the chamber for 1 week at 1 ATA and an airflow of 16 ft/min  $\pm 20\%$  for acclimatization and to check the system's performance. The experiment was initiated at the beginning of the second week without changing airflow or pressure. One week control and experimental periods were alternated. The chamber temperature was maintained at  $28.5^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$  throughout. The experimental air velocities were  $116 \pm 15\%$ ,  $216 \pm 10\%$ , and  $329 \text{ ft/min} \pm 10\%$ . The latter value represents the highest airflow obtainable in this particular system.

On the 2nd, 4th and 7th day of each control and experimental period, all the rats were injected (IP) with  $2 - ^{14}\text{C}$ -acetate (adjusted to pH 7.4 with 80 mM phosphate buffer) at a rate of  $50 \mu\text{Ci/kg}$ . During a 2-hour period beginning immediately after injection, total  $\text{CO}_2$  was trapped at 10-minute intervals in liquid scintillation counting vials containing 0.1% hyamine hydroxide and 0.01% phenolphthalein dissolved in a dioxane-based liquid



scintillation cocktail. The samples were subsequently counted in a L/S spectrometer.

Prior to injection, the animals were weighed and food replaced. The food remaining in the hopper was dried at room temperature for 48 hours and weighed. The difference between the initial and final weights was taken as the measure of food consumption. Water consumption was not quantified.

All data are expressed as per unit of metabolic mass rather than body mass. The use of metabolic mass corrects the data, in part, for the change in body mass and consequently, metabolic rate, of the animals during the course of the experiment. Metabolic mass was calculated by converting individual animal weights ( in kilograms ) to their 0.75 power and summing to obtain total metabolic mass.

### Results:

Under the conditions of this experiment both CO<sub>2</sub> production and O<sub>2</sub> consumption rates increased with an increase in airflow. The greatest change in CO<sub>2</sub> production (8%) was at an air velocity of 329 ft/min. The greatest change in O<sub>2</sub> consumption was a 12% increase above the control level. This, however, occurred at 216 ft/min and the increase in O<sub>2</sub> consumption at 329 ft/min was only 4%. The correlation coefficients between change in CO<sub>2</sub> production or O<sub>2</sub> consumption and air velocity are 0.867 (P<0.01) and 0.558 (P>0.05), respectively. Curves for CO<sub>2</sub> production, O<sub>2</sub> consumption, and RQ plotted against treatment are presented

in Figure 1.

The rate of food consumption (Figure 2) changes in a manner similar to the respiratory data. The greatest change occurred at 329 ft/min, and was 7% above the control level. Lesser changes were observed at 116 and 216 ft/min. The correlation between change in rate of food consumption and airflow was 0.820 ( $P < 0.05$ ).

The rate of change of metabolic mass (essentially weight) also varies in a manner which correlates significantly with air velocity ( $r = 0.751$ ,  $P < 0.05$ ). The greatest change in rate of mass gain, aside from the initial control period, was an increase of 63% at 329 ft/min. A 13% increase in gain rate was observed at 216 ft/min. The change in gain rate at 116 ft/min, although large is negative and appears to be related to events occurring before and during the initial control period. These data are shown in Figure 2.

The production of  $^{14}\text{CO}_2$  from 2- $^{14}\text{C}$ -acetate (Figure 3) declines from the initial control period to the period of highest air velocity, and the rate at which  $^{14}\text{CO}_2$  production decreases over this period is influenced by the air velocity, the rate being lower at increased air velocities. The largest change, 20%, occurred at 329 ft/min. The correlation coefficient of air velocity versus percent change in  $^{14}\text{CO}_2$  production is 0.793 ( $P < 0.05$ ).

The percent change of the metabolic parameters is presented in Table 1. The values represent the change in a given parameter from the

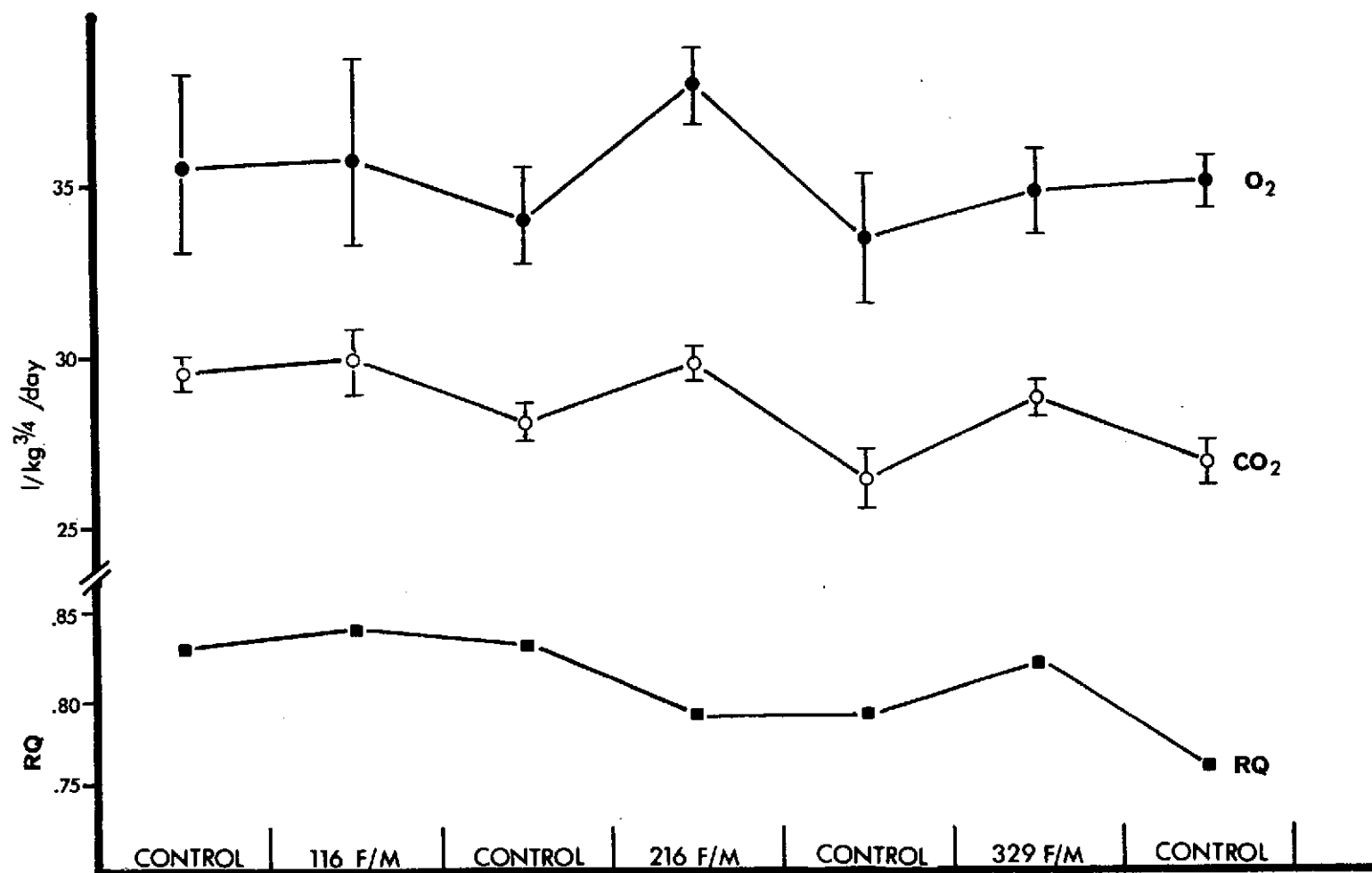


Figure 1. Mean Rate of Oxygen Consumption, Carbon Dioxide Production, and RQ Plotted Versus Treatment Period. Oxygen consumption and CO<sub>2</sub> values on ordinate are expressed in liters per kilogram of metabolic mass per day. Vertical bars are the standard error. Control air velocity is 16 ft/min  $\pm$  20%. Each period is approximately of 7 days duration.

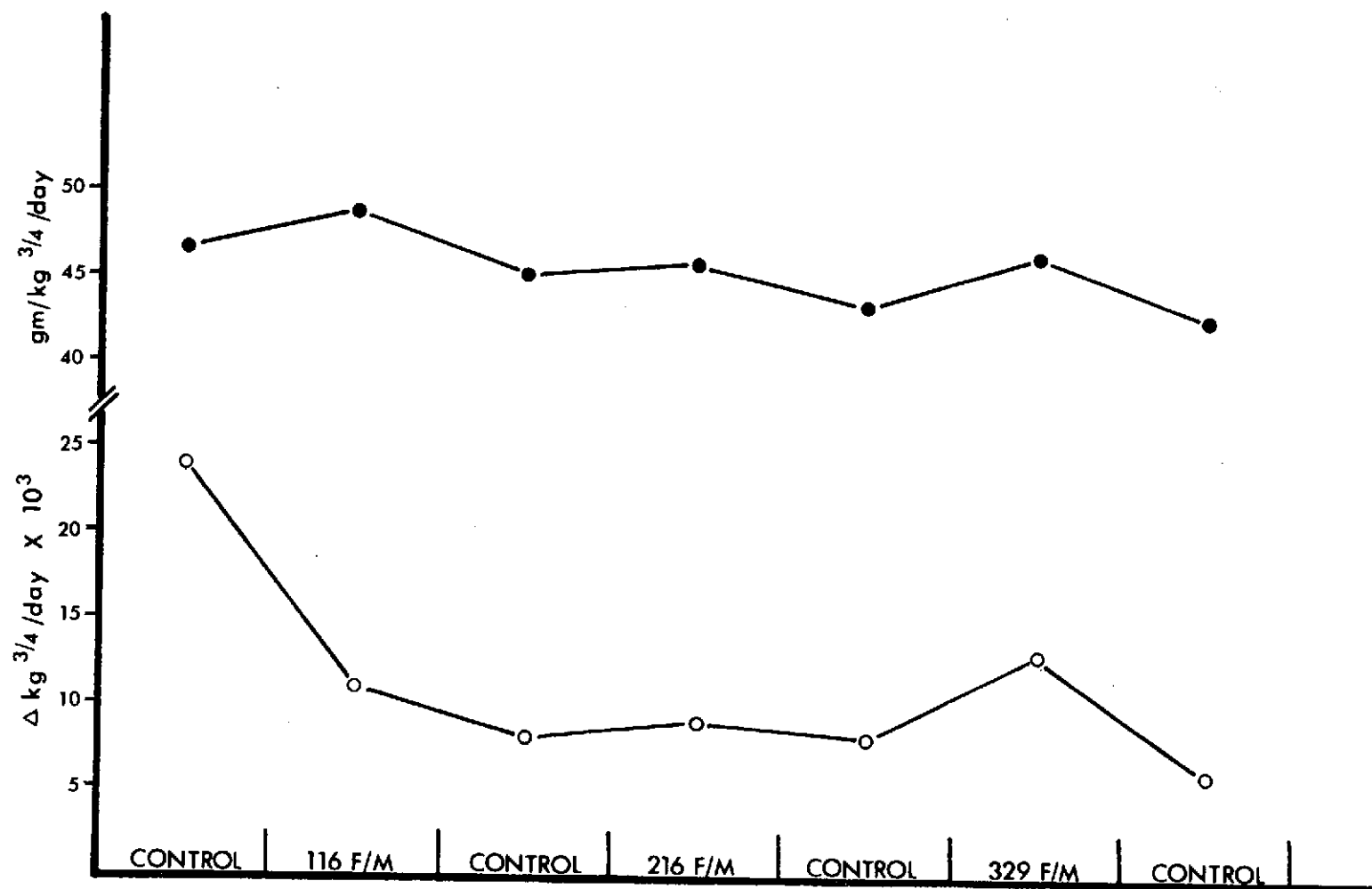


Figure 2. Mean Rate of Food Consumption and Metabolic Mass Gain Plotted Versus Treatment Period. Food consumption is expressed as grams per kilogram of metabolic mass per day while the units for weight gain are kilograms  $^{3/4}$  per day.

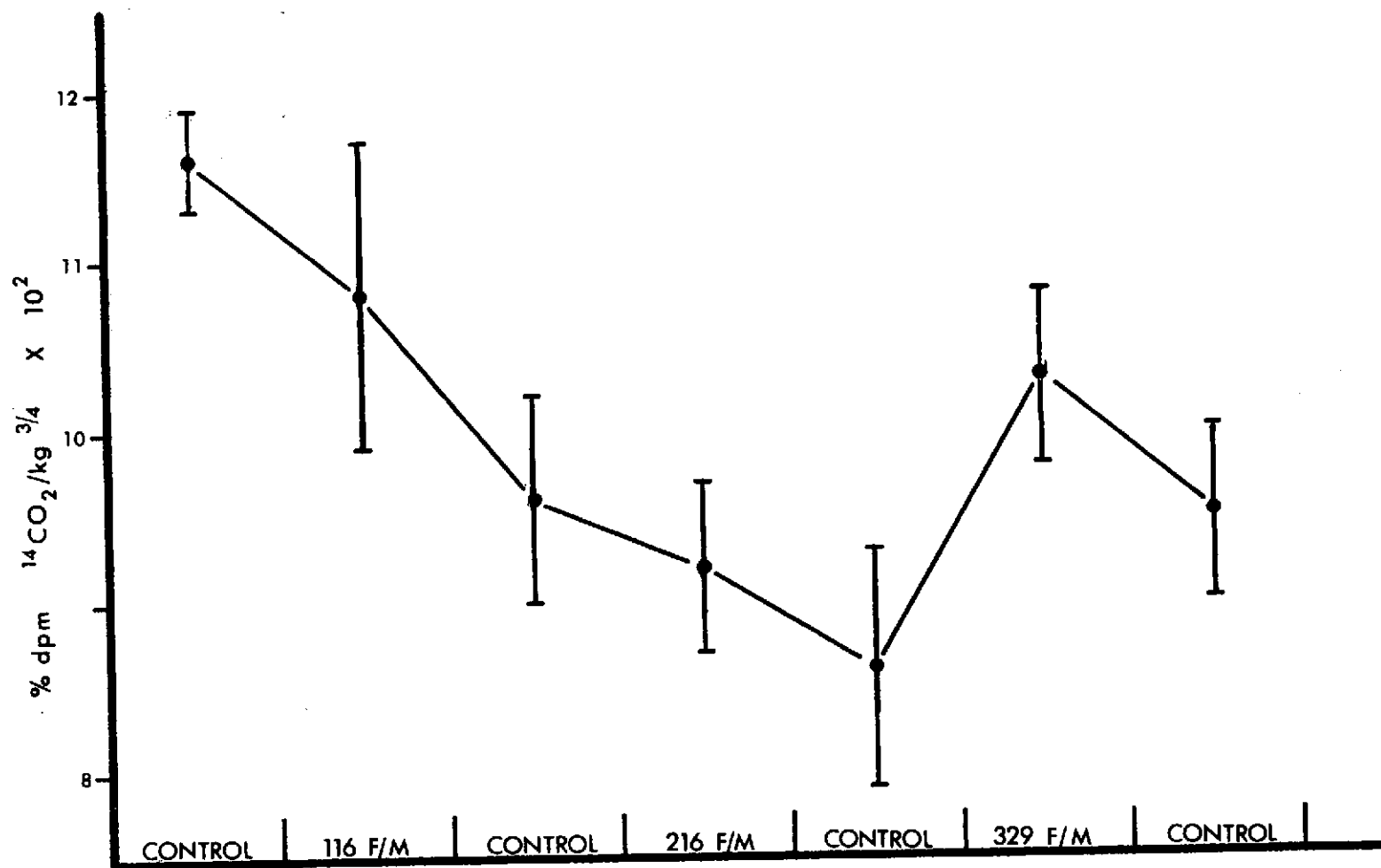


Figure 3. Quantity of  $^{14}\text{CO}_2$  Produced by Rats Following Injection (IP) of 2- $^{14}\text{C}$ -acetate Expressed as the Mean of the Percent uCi Recovered of the Total Amount of uCi Injected per Kilogram of Metabolic Mass. Vertical bars are the standard error.

TABLE I. Percent Change in Metabolic Parameters Relative to Air Velocity.  
 (+ = increase from previous level, and - = decrease from previous level.  
 r = correlation coefficient between metabolic parameter and airflow.)

Airflow	O <sub>2</sub> Consumption $\Delta \%$	CO <sub>2</sub> Production $\Delta \%$	<sup>14</sup> CO <sub>2</sub> Production $\Delta \%$	Food Consumption $\Delta \%$	Rate of Wt. Gain $\Delta \%$
Control*	0	0	0	0	0
116 ft/min	+0.7	+1.5	-7.0	+3.5	-54.0
Control	-5.0	-6.0	-11.0	-7.0	-27.0
216 ft/min	+12.0	+6.0	-4.0	+0.8	+13.0
Control	-12.0	-11.0	-7.0	-5.0	-13.0
329 ft/min	+4.0	+8.0	+20.0	+7.0	+63.0
Control	+0.9	-6.0	-8.0	-8.0	-54.0
r	0.558	0.867	0.793	0.820	0.751
(significance level)	(P > 0.05)	(P < 0.01)	(P < 0.05)	(P < 0.05)	(P < 0.05)

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\* control air velocity = 16 ft/min  $\pm$  20%.

previous level with the initial control period arbitrarily set at 0% change. The correlation coefficients for these data versus air velocity are also shown in this table.

#### Discussion:

The data indicate that chronic exposure to airflow of moderate velocities was stressful. This conclusion derives from changes observed in the metabolic rate and in the apparent oxidized substrate. These effects are most likely attributable to a change in the rate of heat loss, since the variation in several of the metabolic parameters is nearly proportional to the variation in air velocity.

At 116 ft/min both oxygen consumption and CO<sub>2</sub> production increased slightly and were accompanied by a fairly large increase in the rate of food consumption. RQ, however, shifted upward slightly from 0.83 to 0.84 while the rate of metabolic mass gain dropped rather precipitously. The turnover of labeled acetate was similarly enhanced, but was quite small. The data from this period are complicated by difficulties encountered with the water delivery and temperature control systems during the previous two weeks and by the fact that the rats were quite young and in the rapid growth phase. Thus it appears that the principal metabolic activity was directed toward synthesis of fat, structural protein and other materials, the increase in heat loss being offset by a slightly higher rate of carbohydrate oxidation. During the subsequent control period all parameters returned to pre-exposure levels or lower.

On exposure to an air velocity of 216 ft/min, again most parameters were elevated; however, RQ dropped from 0.83 to 0.79. If the difference is significant, it could indicate a greater dependence on the oxidation of lipid or possible proteinaceous substrates. There appeared to be, however, a net synthesis of material since the rate of mass gain increases. It may be more appropriate to refer to this as an increase in the rate of gain of apparent metabolic mass, since it is not possible to estimate the hydration state of the rats during this time. Much of the mass increase could simply be a change in body water. On the return to low air velocity the apparent substrate preference was unchanged and the other parameters returned to levels below those of the pre-exposure period.

Exposure to the highest air velocity, 329 ft/min, elevated the metabolic rate rather dramatically. It is most apparent that the rats were stressed. RQ coupled with other parameters indicates an apparent net synthesis of fat. Although at first this seems incompatible with high rate of acetate oxidation to  $\text{CO}_2$ , one must assume that the flux of acetate through the oxidative pathway is increased in proportion to rate of incorporation into fatty acids. Indeed, on return to control conditions, RQ dropped from 0.82 to 0.76 while  $\text{O}_2$  consumption increases slightly.  $^{14}\text{CO}_2$  production from acetate in this instance did not decrease to the anticipated control level. Thus, it appears that a considerable quantity of lipid was synthesized during the period of high air velocity perhaps to serve as insulation to offset increased heat loss. On return to control air velocity this lipid



material would be an impediment to body temperature regulation and subsequently may be preferentially oxidized.

Heat production, calculated from  $O_2$  consumption and RQ data according to Kleiber (1), during the periods of high air velocity increased from an average of  $152 \text{ kcal/kg}^{3/4}/\text{day}$  to an average of  $160 \text{ kcal/kg}^{3/4}/\text{day}$ , a difference of 5%. The potential rate of heat loss, however, was on the average 4-fold greater ( $3.4$  to  $12.4 \text{ kcal/dm}^2/\text{day}$ ) at the elevated air velocities. The discrepancy between heat production and heat loss is due to the efficiency of the animals' body insulation, e.g. fur and subcutaneous fat.

In summary, it would seem that air velocities up to  $200 \text{ ft/min}$  are without significant effect on the metabolism of rats. An affect becomes apparent at velocities in the neighborhood of  $200 \text{ ft/min}$  and is considerable at higher velocities. In most instances the changes observed in the metabolic parameters were quite small being on the order of 5-10% and were accompanied by large standard errors. Consequently, they are statistically nonsignificant. Additional replication would attenuate the error to a level of acceptable statistical significance.

## IN VITRO EXPERIMENTS ON THE NATURE OF INERT GAS NARCOSIS

### Introduction:

This report details work done during the past few months on the effect of both clinical inhalation anesthetics and argon on forward and reverse electron transport by submitochondrial particles. Additionally, data are presented on the affect of nitrous oxide and argon on firefly luciferase activity. Background literature has been discussed previously in the October 1972 and October 1973 Status Reports.

### Methods and Materials:

The luciferin-luciferase system was prepared from desiccated firefly tails (Sigma). 50 mg of the tails were suspended in 4 ml of 0.1 M sodium arsenate buffer (pH 7.4) at 0°C and sonicated in a 1 M CaCl<sub>2</sub> ice bath for two minutes with a Bronson model W-350 sonifier at an indicated input to the microtip of 75 watts. The sonicate was transferred to a 9 ml polycarbonate centrifuge tube and two 1 ml washings of the sonication vessel were added. It was then centrifuged at 100,000 x g (ave RCF) for 30 minutes at 2°C in a Beckman Type 40 Rotor. The resulting supernatant was brought up to about 6 ml and crystalline MgCl<sub>2</sub> added to a final concentration of 40 mM. This preparation was then stored at 0°C for no longer than 4 hours before use.

Luciferase activity was assayed by transferring 0.5 ml of the supernatant to the high pressure cell, the head space in the cell was

purged with medical grade oxygen, the cell sealed and then pressurized with either helium, argon, or nitrous oxide. Gas was either obtained directly from the cylinder or from a hydraulic pressure accumulator depending on the pressure required. Following a ten minute preincubation period, the reaction was initiated with 10  $\mu$ l of 10.3 mM ATP. The temperature of the cell was maintained at  $22 \pm 0.2^\circ\text{C}$ . Luciferin light emission was monitored with a Beckman DK-2A spectrophotometer, the photomultiplier preamplifier of which was chopper stabilized. The output of the spectrophotometer was recorded on a strip-chart recorder and adjusted so that the initial controls would produce between a 90 and 95% full scale response of the recorder. Light intensity was arbitrarily taken from a point on the intensity decay curve 6-12 seconds after the initiation of the reaction.

The assay of  $\text{NADH:O}_2$  oxidoreductase activity was performed as described in the October 1973 Status Report. The procedure for assaying succinate:NAD oxidoreductase activity was inadvertently omitted from the previous report and is now described. An aliquot containing up to 300  $\mu\text{g}$  of submitochondrial particle ( $\text{ETP}_p$ ) protein is added to a volume of assay medium containing 40 mM triethanolamine buffer (pH 7.8), 6 mM  $\text{MgCl}_2$ , 1.5 mM KCN, and 0.3 M mannitol. Succinate and NAD are then added to a final concentration of 5 mM and 1 mM, respectively. The reaction is initiated by addition of ATP to a final concentration of 3 mM. The change in absorbance at 340 nm and  $25^\circ\text{C}$  is followed on a strip-chart

recorder and the rate of change is calculated from the linear portion of the curve.

The studies of these enzyme activities as affected by some of the clinical inhalation anesthetics was repeated using a procedure somewhat different than outlined in October 1973 Status Report. Rather than adding the anesthetic directly to the assay medium, the assay medium was equilibrated with the anesthetic in the gas phase. Air was bubbled through a small vaporizer, maintained in a constant temperature bath and mixed on the effluent side with additional air to produce the desired concentration of anesthetic. The mixture was passed over a 25 ml volume of assay medium in a 50 ml filter flask at 25°C. Anesthetic concentration of the gas mixture was periodically assayed by gas chromatography using an H-P model 7620 gas chromatograph equipped with flame ionization detectors. The anesthetic was resolved with 3% OV-17 on Chromsorb W(HP) 80/100 mesh in a 1/8" x 8' stainless steel column at 100°C. A standard curve was prepared by sampling the headspace of a sealed container of the anesthetic maintained at a constant temperature.

Aliquots of an ETP<sub>p</sub> suspension were added to 1 ml volumes of the anesthetic-equilibrated assay medium in a 1.2 ml quartz cuvette and the reaction initiated within 1 minute by the addition of the appropriate substrate. The change in absorbance at 340 nm was followed at 25°C with a Beckman DB-G spectrophotometer. Anesthetics used were halothane (Ayerst), methoxyflurane (Pittman-Moore), and diethyl ether (Mallinckrodt).

The diethyl ether was extracted with 5% aqueous ferrous sulfate and dried over anhydrous sodium sulfate prior to use.

Assay of ETP<sub>p</sub> enzymatic activity at various pressures of argon and nitrous oxide were conducted at 25°C as described for the luciferase assay.

### Results:

Both halothane and methoxyflurane were observed to be rather potent inhibitors of NADH:O<sub>2</sub> oxidoreductase. The ED<sub>50</sub> (effective dose required to produce a 50% inhibition of activity) concentrations for halothane and methoxyflurane were 1.95 and 0.38%, respectively. On the other hand, 50% inhibition of forward transport was not realized with concentrations of diethyl ether up to 9.6% at which the inhibition was 26%. Complete inhibition of forward electron transport was not observed with either halothane or methoxyflurane at concentrations of up to 4%. The susceptibility of reverse flow, i.e., succinate:NAD oxidoreductase was tested only with methoxyflurane. The ED<sub>50</sub> was estimated to be less than 0.1%. (Difficulty was encountered in accurately determining the concentration of methoxyflurane below 0.3%). Data for these anesthetics is shown in Figure 1.

The affect of argon at pressures up to 120 ATM was negligible. Although some inhibition was observed (between 2 and 5%), it was not reproducible and assays with helium up to 100 ATM produced about the same results. (It should be stressed that the overall error for this

particular assay is on the average  $\pm 4\%$ .) Assays conducted with  $N_2O$  on the other hand, were somewhat more encouraging. Foreward electron transport was completely inhibited at 40 ATM  $N_2O$ ; the  $ED_{50}$  for this anesthetic is about 18 ATM (Figure 2).

The effect of argon on reverse electron transport was evaluated at pressures up to 120 ATM. The action of this gas was rather bizarre. At 50 and 75 ATM reverse transport appears to be activated slightly returning to control activity above 75 ATM and inhibited approximately 12% at 120 ATM (Table 1). This behavior currently appears to be reproducible. However, pressure controls with helium have not been performed and consequently it is not certain whether this is an effect of argon or pressure. The effect of  $N_2O$  on reverse transport has not been tested.

As observed with foreward electron transport, the activation of firefly luciferin by luciferase is significantly inhibited by  $N_2O$  (Figure 2); the  $ED_{50}$  is slightly higher than that for  $ETP_p$ : 23 ATM. Unlike foreward electron transport, complete inhibition was not observed at 40 ATM. Contrary to the response of the respiratory chain, luciferase is inhibited by argon at pressures of 50 and 100 ATM (Figure 2). The effect of helium on luciferase activity at these pressures is also presented in this figure.

#### Discussion:

The results reported here for halothane and methoxyflurane differ considerably from those reported for the same anesthetics in the October

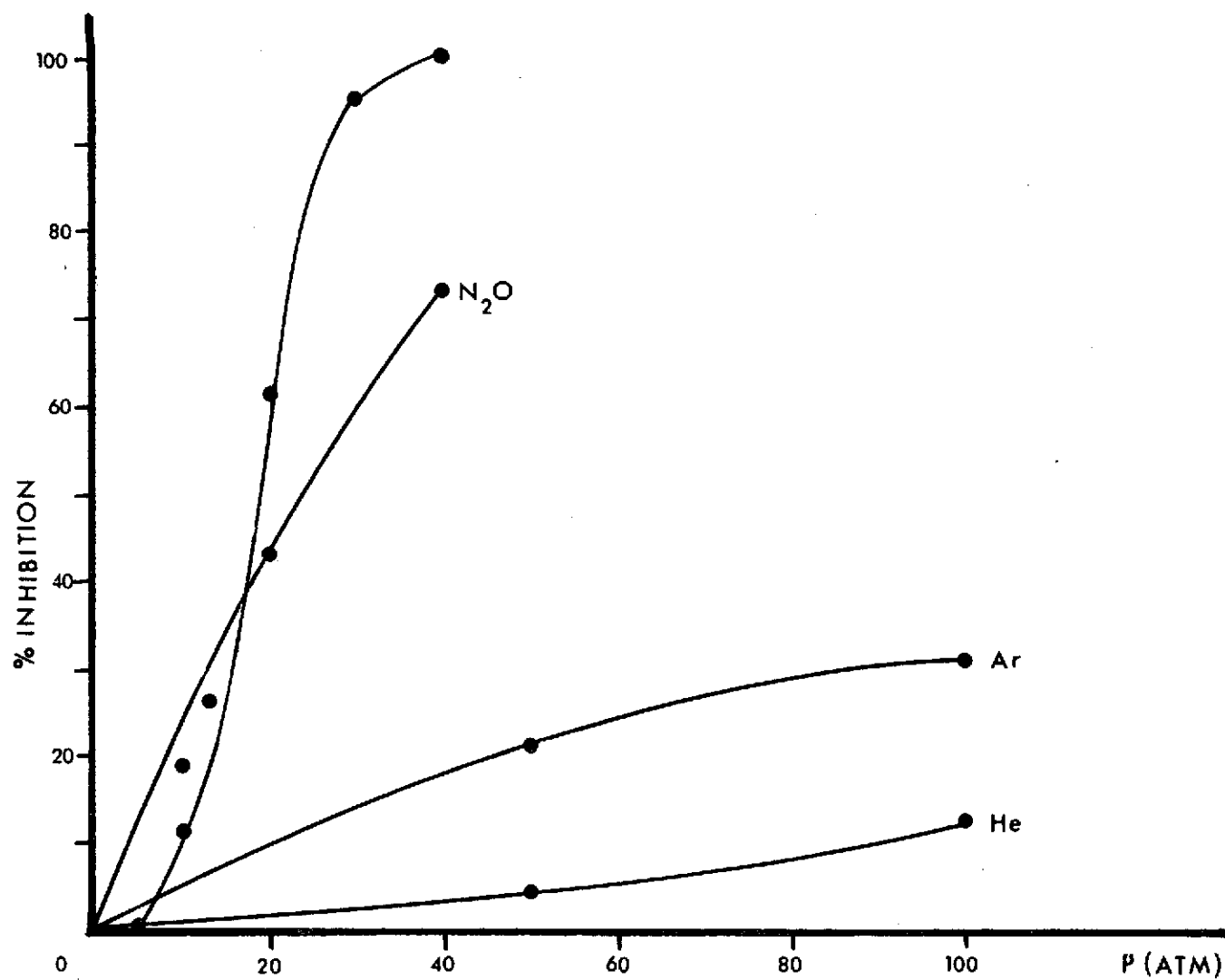


Figure 1. Per Cent Inhibition Versus Concentration of Anesthetic in Air. (% v/v)  
 1) succinate:NAD oxidoreductase and methoxyflurane; 2,3,4) NADH:O<sub>2</sub> reductase and methoxyflurane, halothane, and diethyl ether, respectively.

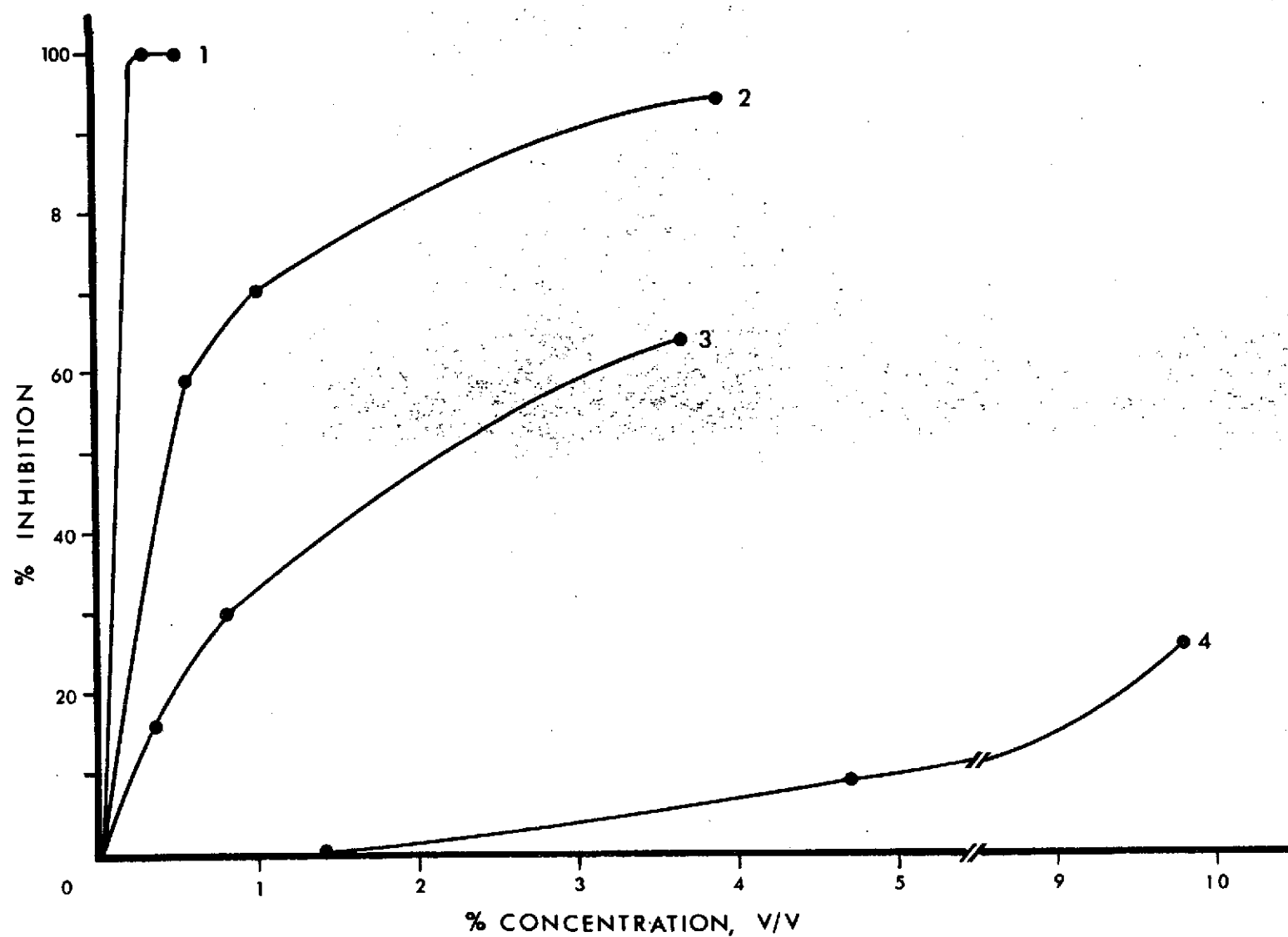


Figure 2. Inhibition of ETP<sub>D</sub> NADH Oxidase (upper most curve) by Nitrous Oxide and the Inhibition of Firefly Luciferase by Nitrous Oxide, Argon, and Helium (lower curves).



Table I. Effect of Argon at Pressure on the Activity of Succinate:NAD Oxidoreductase. Assays were performed as described in text.

<u>CONDITIONS</u>	<u>n</u>	<u>Velocity**</u>	<u>% Inhibition</u>
Control*	10	0.045 $\pm$ 0.002	0
Argon, 50 ATM	5	0.048 $\pm$ 0.001	-6.6
75 ATM	4	0.047 $\pm$ 0.001	-4.4
Control	7	0.040 $\pm$ 0.002	0
Argon, 120 ATM	4	0.035 $\pm$ 0.002	+12.5

\* Control assays were run at ambient pressure in the absence of argon.

\*\* Velocity = mean  $\Delta A_{340}/\text{min} \pm$  standard error.

1973 Status Report. The latter data indicated  $ED_{50}$ 's of about 1.5 mM and 2 mM for methoxyflurane and halothane, respectively, and contrast with the 0.67 mM (0.35%) obtained for methoxyflurane and 1.2 mM (1.95%) more recently determined. The current data are in better agreement with the  $ED_{50}$ 's reported by Cohen and McIntyre (2) of 0.59% (methoxyflurane), 1.35% (halothane) for the inhibition of glutamate oxidation by intact rat liver mitochondria and calculated halothane  $ED_{50}$ 's of 1-2% for 3-hydroxybutyrate-mediated swelling of rat liver mitochondria (3). These may be compared with the in vivo determined minimum alveolar concentrations (MAC) for halothane of 0.87% and for methoxyflurane of 0.23% (4), although one should understand that the in vitro and in vivo endpoints may not be identical because of the differences in the biological systems involved.

The current data for the inhibition of NADH oxidase activity by diethyl ether diverge considerably from the anticipated result. Cohen and McIntyre observed an  $ED_{50}$  for this anesthetic of 7.9% for the mitochondrial oxidation of glutamate with nearly complete inhibition above 11%. The reported MAC for diethyl ether is 3% (4) and although Cohen's value is more than 2-fold above this value, it is in better agreement with the MAC than the  $ED_{50}$  value of 18% extrapolated from our data. Harris, et al (5), however, reported that a concentration of 340 mM ether, which is equivalent to a 30% gas phase concentration, was required to effect a 94% inhibition of NADH oxidation by submitochondrial particles prepared from bovine heart muscle. A linear extrapolation of our data to a 30%

gas phase concentration of ether would similarly predict a better than 90% inhibition of NADH oxidase activity. Even though such an extrapolation is not entirely proper, because of the hyperbolic shape of the inhibition curve, it does indicate that our data are comparable to Harris'.

The discrepancies in these data may be attributed to several reasons. Principal among these is that Cohen utilized intact mitochondria oxidizing a NAD-linked substrate as opposed to the system used by Harris and us in which the oxidation of NADH was followed directly. One could argue that diethyl ether interferes inhibitorily with the movement of glutamate into the mitochondria and its subsequent oxidation to oxoglutarate as well as the oxidation of NADH. These are problems more exclusive to intact mitochondria than submitochondrial particles. It is known that both ether and methoxyflurane at moderate concentrations significantly inhibit the oxidation of succinate by intact rat liver mitochondria, but they do not affect the oxidation of this substrate by osmotic-shock prepared submitochondrial particles (4). Higher concentrations of ether than those used by Cohen, however, can inhibit the succinate oxidase activity of submitochondrial particles (5). It is not clear whether there is any similarity in the mechanism(s) of succinate oxidase inhibition in these two types of preparations.

Another, but less likely possibility for the variance in the ether data, is the manner in which the mitochondria and submitochondrial particles were incubated with the anesthetic. Cohen pre-incubated rat

liver mitochondria with ether (in the gas phase) at 0°C. We, however, pre-incubated the submitochondrial particles at 25°C with an assay medium which had been equilibrated with this anesthetic at the same temperature. Harris' procedure was essentially the same as ours, although the manner in which the anesthetic was introduced to the assay medium differed slightly. As a consequence of the much higher solubility of ether in aqueous solutions at 0°C than at 25°C it is possible that its concentration was significantly higher than estimated by the gas phase concentration. The reason this seems to be an unlikely factor is apparent when our data for halothane and methoxyflurane are compared with Cohen's. The agreement is very good. Because of the differences in the assay systems, the effect of pre-incubation temperature cannot be fully evaluated.

The effect of nitrous oxide on the rate of NADH oxidation by submitochondrial particles was rather startling in view of the anesthetic potency of this gas (MAC 90%). The ED<sub>50</sub> for nitrous oxide calculated from our data is about 18 ATM or 20-fold greater than the comparable in vivo concentration. But this is close to the 14 ATM of nitrous oxide required for 60% inhibition of either frog epithelial sodium transport or sciatic nerve conduction (10). Nahrwold (11) found that a concentration of nitrous oxide of 0.65 ATM did not inhibit the oxidation of NAD-linked substrates by rat liver mitochondria. However, when combined with various concentrations of halothane the effect of nitrous oxide was entirely additive. No explanation was given for these results.

Earlier Nahrwold and Cohen (12) empirically derived an equation to predict the ED<sub>50</sub> values of various anesthetics based on their oil/gas partition coefficients and concentrations required for 50% inhibition of NAD-linked substrate oxidation:

$$\log_{10} \text{ED}_{50}(\%) = 2.746 - 1.035 \log_{10} \text{O/G}$$

where O/G is the oil/gas partition coefficient. This equation, which predicts an ED<sub>50</sub> of 4 ATM for nitrous oxide, has been successfully used to predict the synergistic effect of mixtures of nitrous oxide and halothane in various ratios (11). The calculated N<sub>2</sub>O ED<sub>50</sub> is obviously at some variance with our data.

Similarly, when this equation is used to predict the ED<sub>50</sub> of argon a value of 41.5 ATM is obtained. Since pressures of argon up to 3 times this value were without significant effect on NADH oxidation, we feel this equation does not fully describe inhibition of NADH oxidation by, presumably, active agents. A portion of the error could be attributed to the differences in the biological assay materials: Nahrwold's equation is based on assays with intact mitochondria, while our assays are performed with sonically derived submitochondrial particles.

The greatest probable source of error is in the derivation of the equation; it is based entirely on data for only six inhalation anesthetics. In this light it is not likely to successfully predict the behavior of anesthetics whose solubilities and molecular volumes deviate greatly from those on which the equation is based.

Using the ED<sub>50</sub> for nitrous oxide, however, we calculated the ED<sub>50</sub> for argon to be approximately 150 ATM, which approximates the 163 ATM required to block frog sciatic nerve conduction (10). This calculation, unlike Nahrwold's, takes into account the differences in aqueous and membrane solubilities between these two gases as well as the difference in their molecular volumes, all of which have significant bearing on their relative effectiveness (13).

The difference between nitrous oxide and argon with respect to membrane solubility is not as great as would be predicted by their difference in oil solubility, a factor of 1.5 as opposed to 11, since the latter fails to account for the presence of proteins and other non-lipid materials commonly found in biological membranes. Furthermore, the oil solubility coefficient does not account at all for their solubility in an aqueous system, which is significant here because we are considering a three phase system: gas, aqueous solution and a lipid/protein suspension, and they differ by about a factor of 7. In addition to relative solubilities there is a factor of 1.7 difference in their molecular volumes. The significance of molecular volume in predicting relative anesthetic potencies was suggested by Mullins (14) and later extensively verified by Seeman (13). In essence, Mullins' theory predicts that the efficiency of an anesthetic is determined not only by its solubility, but on the volume it occupies as well. Thus, assuming two compounds have the same solubilities, the one with the larger molecular volume will, on the average, be the more effective.

The data compiled by Seeman for the most part support the validity of this theory.

Our data agree better with calculated membrane concentrations not corrected for molecular volume than those in which this correction has been made. Our very small sample of anesthetics, however, makes an extrapolation to a general model somewhat difficult. This is evidenced by the fact that we did not observe any inhibition of NADH oxidase at 120 ATM of argon, a value which is close to the calculated  $ED_{50}$ . We currently feel that the high  $ED_{50}$  for  $N_2O$  and the apparent lack of affect on NADH oxidation by argon are attributable to factors not totally accounted for by mathematical solutions encompassing various solubility factors and molecular volumes.

One factor commonly not considered in such calculations is the change in the chemical activity with pressure. From a series of measurements on aqueous solutions of various gases among which were argon, nitrogen and helium, Enns, et al (15) reported that on the average the activity was increased by 14% at a pressure of 100 ATM. This means that the apparent concentration of a gas in solution may be 14% higher than the true concentration. Concentration errors at lower pressures are less. Thus, at a pressure of 120 ATM argon the effective pressure would be slightly greater than 100 ATM.

To realize the calculated  $ED_{50}$  for argon of 150 ATM, a pressure of 200 ATM would be required. This is assuming that the  $ED_{50}$  for

nitrous oxide is in error by no more than 3% with regard to its true concentration. The increase in activity may well explain the absence of significant inhibition of NADH oxidase at high pressures of argon, but it will not satisfactorily explain the high  $ED_{50}$  for nitrous oxide; at 40 ATM the error would only be about 6%. In lieu of other explanations the apparent anomalies are considered to be more biological than physical in nature.

The complete cessation of succinate:NAD oxidoreductase activity occurred at a gas phase concentration of methoxyflurane approximately an order of magnitude below that required for comparable inhibition of NADH oxidase. There are two explanations for this apparent low tolerance: 1) the reverse reaction being thermodynamically unfavorable, the possible disorder effected by the presence of the anesthetic in complex I may raise the energy requirement for the transport of electrons, and 2) the anesthetic may interfere with either the hydrolysis of ATP or the coupling of the energy derived from this hydrolysis to complex I.

Harris (5) is of the opinion that a reduction of energy coupling to complex I is not responsible for the inhibition, but rather is a reduction of the enzymatic activity. Indeed, both Miller (3) and Rosenberg (6) observed no change in either ADP/O or P/O ratios at concentrations of halothane below 2-3%. Similarly, Cohen (2,7) reported that the uncoupling of rat liver mitochondria occurred only above concentrations of 0.64% and 2% of methoxyflurane and halothane, respectively. However, halothane



significantly inhibits energy-linked mitochondrial swelling with either succinate or 3-hydroxybutyrate as a substrate (3) as well as the ATP- or succinate-driven calcium uptake by rat brain mitochondria (6) at concentrations less than half of the  $ED_{50}$  for forward transport. Although one might argue that the anesthetics, in the case of intact mitochondria, interfere with substrate transport, the oxidation of succinate is not hindered by concentrations of methoxyflurane or succinate well above their forward transport  $ED_{50}$ 's (3,7). Additionally, the  $ED_{50}$  for reverse flow calculated from Harris' data yields a value which is similarly less than half the forward transport  $ED_{50}$ . In accordance with these data the reverse flow  $ED_{50}$  determined for the current data indicates a value between one-half and one-third of the forward flow  $ED_{50}$ . Finally, Snodgrass and Piras (8) found that dinitrophenol-stimulated mitochondrial ATPase was inhibited at halothane concentrations below those producing uncoupling. This is in accordance with the observed reduction of mitochondrial ATP- $P_i$  exchange in the presence of amytal (9).

Even though there are some significant problems involved in making comparisons between different types of mitochondrial preparations, it appears that the inhibition of reverse electron transport is more closely related to inhibition of energy transduction than to the enzymatic activity of complex I. A more rigorous study of the inhibition of this enzyme complex by various clinical inhalation anesthetics will have to be conducted before a more definitive conclusion can be drawn.

Since we failed to detect significant inhibition of NADH oxidation by argon, we turned to succinate:NAD oxidoreductase in the belief it would be more sensitive to environmental perturbation than the forward reaction. Below 100 ATM reverse transport appears to be activated while above it is inhibited. The degree of activation or inhibition, however, is small, and the variation exceeds 2 standard errors of the mean. Consequently, these changes are statistically nonsignificant.

If the apparent activation is real rather than artifactual it would be interesting to determine its relationship to conformational changes believed to occur in submitochondrial particles on energization (15,16). The activation phenomenon is most likely due to pressure while inhibition is probably attributable to argon. However, these effects will have to be characterized more extensively with  $N_2O$ , helium and krypton.

The effect of nitrous oxide and argon at pressure on the activity of firefly luciferase, was investigated, because this enzyme represents a very simple system relative to the respiratory chain and is sensitive to environmental perturbation, e.g. temperature, pressure, and hydrophobic agents. It also represents a non membrane system on which to test the effect of inert gases. Ueda (17) has shown that luciferase activity is reduced in the presence of several clinical inhalation anesthetics in a manner which correlates with their solubility. He equated his results to the theory of absolute reaction rates developed by Eyring and applied

to bacterial luminescence by Eyring and Magee (18).

As applied to luciferase the theory, in essence, dictates that inhibition occurs either by direct interaction of the inhibitor with the active site(s) of the enzyme or by accelerating the conversion of the enzyme from the active to inactive state (involving a conformation change). The former is designated a type I inhibitor while the latter is type II. In regard to the latter, Brown, et al (19) observed an increase in the apparent volume of bacterial luciferase on conversion from the active to inactive form. Johnson, et al (20) reported that the inhibitory effect of hydrophobic compounds such as alcohol and urethane could be reversed by hydrostatic pressures of several hundred ATM. From these data Eyring and Magee concluded that certain hydrophobic agents accelerate the formation of the inactive form of the luciferase by filling a hydrophobic space in the enzyme and thereby preventing its conversion to the active form.

Ueda's data demonstrates that the inhibition of luciferase occurs as a type II process. He reported large negative changes in enthalpy and entropy which approximately correlate with the oil solubility coefficients of the anesthetics. He interpretes this behavior indicative of a conformational change or unfolding of the enzyme.

Both nitrous oxide and argon were observed to inhibit firefly luciferase in a manner significantly correlated with their concentrations corrected for solubility ( $r=0.969$ ,  $P<0.01$ ). These data are not

sufficient to determine whether a type I or type II inhibition is involved, since data at several temperatures is required. However, in view of the correlation between inhibition and solubility it is currently assumed that these data would fit the type II model.

The inhibition curve for helium is quite puzzling. It was believed that these data represented an inhibition of luciferase by pressure, however, Brown et al (19) indicate that pressure effects, within the range of pressures used here and at the optimum temperature for the enzyme, are negligible. In fact, one would expect luciferase activity to increase with pressure rather than decrease, because the rate of conversion to the inactive state would be reduced. Consequently, more of the enzyme would be in the active form. Inhibition is less likely to be attributable to helium, because of its very low solubility, more than an order of magnitude below argon. Until this effect is further characterized, we currently ascribe it to a contamination of helium with some active compound, e.g. nitrogen.

In summary, we have found that both methoxyflurane and halothane inhibit NADH oxidation by submitochondrial particles to about the same degree as the inhibition of oxidation of NAD-linked substrates by intact mitochondria. The inhibition of NADH oxidase by diethyl ether is considerably less in submitochondrial particles than would be predicted by the behavior of mitochondria. This may be due to either structural or substrate differences, although the differences in methodology may also be

significant.

The inhibition of  $\text{ETP}_p$  NADH oxidase by nitrous oxide occurred at pressures 5-fold greater than that predicted from its effect on intact mitochondria. On the other hand, argon at pressures in considerable excess of the predicted  $\text{ED}_{50}$  were without effect on NADH oxidase. These discrepancies are believed to be caused by the inadequacies of the prediction model.

Methoxyflurane inhibited reverse electron flow at much lower levels than required for comparable inhibition of forward flow. A reduction of complex I enzymatic activity or energy transduction would explain this phenomenon. Argon may inhibit reverse flow at pressures above 120 ATM, but below this pressure it appears to activate this reaction.

Firefly luciferase was inhibited by both argon and nitrous oxide. The inhibition of luciferase by  $\text{N}_2\text{O}$  was quite similar to its inhibition of NADH oxidase. The activity of luciferase was also depressed by argon up to pressures of 100 ATM, and demonstrates that membrane structures are not a prerequisite for the action of an inert gas. The difference in the amount of inhibition caused by these gases is proportional to their lipid solubilities.

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## APPENDIX

The following paper entitled, "A Rapid Quantitative Measure for Comparative Metabolic Activity", by J.P. Jordan, C.L. Schatte, D.P. Clarkson, M.L. Corrin, W.W. Martz and C.L. Clow has been prepared for submission to the editors of Comparative Biochemistry and Physiology. It is an expanded and up-dated version of work presented in an earlier Status Report.



A RAPID QUANTITATIVE MEASURE FOR COMPARING METABOLIC  
ACTIVITY

by

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# A RAPID QUANTITATIVE MEASURE FOR COMPARING METABOLIC ACTIVITY

## Abstract

A quick, simple measure of metabolic activity by measuring the conversion of a  $^{14}\text{C}$ -labeled substrate ( $\text{C}_0$ ) to  $^{14}\text{CO}_2$  (P) is described. A plot of  $\ln (\text{C}_0 - \text{P})$  versus time provides a readily quantified parameter sensitive to both changes in metabolic rate and the nature of the substrate oxidized. Although acetate-1- $^{14}\text{C}$  was the substrate most frequently used, labeled glucose and pyruvate have also been employed. The procedure involves only an initial injection of labeled substrate followed by collection of expired  $^{14}\text{CO}_2$  for approximately 60 minutes. The animal is not anesthetized, restrained, or starved. The technique correlates well with the standard but more difficult measurement of oxygen consumption.

metabolic rate; radioisotope kinetics; expired  $^{14}\text{CO}_2$

## Introduction

Because of the definitive nature of mathematics, biologists have long sought to use it as a tool for quantifying changes in animal metabolism. While classical metabolic parameters such as oxygen consumption and carbon dioxide production are used as whole body metabolic rate indicators, it is advantageous to measure substrate level metabolic activity in order to increase the sensitivity of measurements of metabolic aberrations in the whole animal. Such a parameter must be quickly and easily measured, readily quantified for comparative purposes, and cause minimum disturbance to the animal. The development of such a parameter has been crucial to work in this laboratory which is concerned with long-term biochemical adaptation of rats to artificial environments. The need to maintain as unperturbed a state as is possible while performing repeated measurements over periods of several weeks has prompted us to search for a cellular level indicator that can be repeatedly and reproducibly used while leaving the rats physiologically unaffected.

We have found that the injection of acetate-1- $^{14}\text{C}$  and treatment of the resulting  $^{14}\text{CO}_2$  expiration curve as a simple exponential function provides us with an accurate measure of tricarboxylic acid (TCA) cycle activity (and, therefore, a major part of cellular oxidative activity) which is readily quantified for comparisons between different groups of animals. The sampling time can be as little as 60 minutes, radioactive analysis

requires no chemical treatment, and reproducibility can be as good as 5%. Except for an initial intraperitoneal injection, the animals are unrestrained, unstarved, uncatheterized, and essentially undisturbed in the environment to which they are exposed.

The measurement of oxygen consumption, carbon dioxide production and heat output have long been the standard means of assessing metabolic rate of organisms. The use of various carbon-14-labeled compounds can provide additional information concerning metabolism at the cellular level. However, the usefulness of this information depends on the precise quantification of expired  $^{14}\text{CO}_2$  data such that changes in metabolic rate at the cellular level can be measured with good sensitivity.

We have applied a mathematical treatment to expired  $^{14}\text{CO}_2$  data in order to determine whether or not the sensitivity of such data could be increased above that attendant upon standard integration of expired  $^{14}\text{CO}_2$  curves. When interpreted in conjunction with other metabolic parameters, the procedure provides an additional measure of metabolic rate plus insight into possible biochemical changes which may occur even in the absence of a change in metabolic rate.

## Methods

In a series of twenty-five experiments, groups of rats were exposed to artificial environments in a chamber system which has been previously described (1,2) for periods of three days to thirteen weeks. At intervals of one to seven days throughout the exposure, groups of 4-12 rats were injected intraperitoneally with 25-150  $\mu\text{Ci/kg}$  of sodium acetate-1- $^{14}\text{C}$  in 0.3 - 0.5 ml distilled water. The expired gas was bubbled through a carbon dioxide scrubber containing 10 liters of 3N KOH. The contents of the scrubber was sampled for periods of 1-24 hours at intervals of 10 minutes for the first hour post-injection, 20 minutes during the second hour, and 20, 30 or 60-minute intervals thereafter. Activity of the samples was determined by pipetting 0.1 ml of the KOH solution into 15 ml of p-dioxane - PPO - POPOP - naphthalene - ethanol - Cab-O-Sil\* and counting in a liquid scintillation spectrometer for three 5-minute counts each. A typical plot of the accumulative disintegrations per minute versus time is shown in Figure 1. The shape of the curve and its mathematical analysis have been rigorously treated by others (6,7,8) as conforming to the general equation:

$$A = A_1 e^{k_1 t} + A_2 e^{k_2 t} + A_3 e^{k_3 t} \quad (1)$$

where A = activity in the expired  $\text{CO}_2$  at a given time t

$A_n$  = constant coefficient-intercepts of the individual peeled components (n=1,2,3)

$k_n$  = constant values which represent the slope of the individual peeled components (n=1,2,3)

In equation 1 generally  $A_1 e^{k_1 t}$ ,  $A_2 e^{k_2 t}$  and  $A_3 e^{k_3 t}$  are referred to respectively as the fast, intermediate and slow components which make up the equation.

We have found that it is only necessary to analyze the fast component in order to get a quantitative measure of the metabolic activity of the rat. An additional fact which supports the above is that we have found that factors likely to influence the shape and time course of the expired  $^{14}\text{CO}_2$  curve do not vary appreciably in our experiments (i.e. bicarbonate pool size, cellular uptake of  $\text{CO}_2$ , exchange with bone). A satisfactory interpretation has resulted by expressing the data as:

$$\ln(^{14}\text{C injected/kg} - ^{14}\text{C expired/kg}) = \ln C_0 = kt$$

where  $k$  is the rate of decrease in total body activity per kilogram of rat,  $t$  is time and  $C_0$  is the total activity in the pool at time zero. There is a ten minute dead time for our system. The dead time consists of the events between injection of the substrate and its appearance as  $\text{CO}_2$  in the scrubbers. These include absorption into the blood, distribution to the cells, metabolic conversion, transport to the lungs and expiration as  $\text{CO}_2$ , equilibrium with the chamber gas, and transport to and equilibrium with the scrubber contents.

We have consistently obtained a good bit of the data for the fast component which covers a period of about 90 minutes. Thus, a sufficient sampling period was available over the time interval 10-90 minutes in order to calculate the slope and intercept of the intermediate curve.

Additionally, both our data and the data of others (3,6) have indicated that a substantial fraction of injected acetate is expired during a period of one or, at most, two hours post injection. Subsequent elimination of label from the body does not appear to significantly affect the results.

The slope of the logarithmic plot was determined by best fit of a least squares regression analysis (Figure 2). The resulting value,  $k$  or rate constant, was used for quantitative comparisons among different experimentally-treated groups of animals.

To determine the relationship of this technique to the standard measurements of oxygen consumption or the ratio  $\frac{\text{dpm expired}}{\text{dpm injected}} \text{ kg}^{-1}$ , ten adult, male hamsters were exposed to various treatments in the chamber system. Exposure periods were for 72 hours during which oxygen consumption was continuously measured over six-hour periods.

All exposures were in air at ambient pressure (5000 ft., 632 mm Hg) at 22°C unless otherwise indicated. At the end of the exposure period, the animals were injected with sodium acetate-1-<sup>14</sup>C and the expired CO<sub>2</sub> trapped in 3N KOH scrubbers. The scrubbers were sampled at 5 minute intervals during the first hour and every 10 minutes during the second hour post injection. One-tenth ml of each sample was pipetted into 20 ml of p-dioxane-PPO-POPOP-naphthalene-ethanol-Cab-O-Sil cocktail and counted in a liquid scintillation spectrophotometer for three 10-minute counts each.

The effects of the number of animals injected (2, 5 or 10), the dosage (25, 50 or 100 uCi/kg made up to 25, 50 or 100  $\mu$ Ci/ml distilled water)

and various treatments designed to alter metabolic rate were examined. The treatments included exposure to argon-oxygen (4:1), nitrous oxide-oxygen (4:1), barotrauma immediately prior to injection (decompression from 5000 ft. to 20,000 ft. to 0 ft. to 5000 ft. at 1000 ft/min.), cold exposure (12°C ambient and contamination of the chamber with argon immediately prior to injection.

The activity data were corrected to  $\frac{\text{dpm expired}}{\text{dpm injected}} \text{ kg}^{-1}$  and the logarithm plotted as a function of time. The resulting exponential curve can be described by the equation (9):

$$A(t) = A(o)e^{-kt}$$

where:  $A(t)$  = activity of  $^{14}\text{CO}_2$  at time  $t$

$A(o)$  = activity of  $^{14}\text{CO}_2$  at  $t = 0$

$k$  = rate constant equal to the slope of the curve

The slope  $k$ , which was determined by linear regression, was taken as a qualitative assessment of the rate of  $^{14}\text{CO}_2$  expiration and, indirectly, as a measure of  $\text{CO}_2$  production at the cellular level. These values were correlated with the oxygen consumption during the final six hours prior to injection and compared to the correlations between oxygen consumption and integration of the expired  $^{14}\text{CO}_2$  curve which was obtained by summing the total dpm expired during the collection period.



## Results

As shown in Figure 3, the value of  $k$  will vary in magnitude as a function of the catabolic rate of the injected substance; the greater the rate, the greater the value of  $k$ . This particular set of curves represents the conversion of acetate-1- $^{14}\text{C}$  to  $^{14}\text{CO}_2$  in rats exposed to neon-oxygen for 4 weeks as compared with air controls and was calculated from data previously published (4). In this case, acetate oxidation to  $\text{CO}_2$  mirrored both total metabolic rate and the activity of the tricarboxylic acid cycle; on that basis, the results graphically illustrate metabolic adaptation to the neon environment for the four-week period.

The half-life of the metabolic pool can be determined by:

$$t_{1/2} = \frac{\ln 2}{k}$$

If the injected substrate mixes with the appropriate substrate pool(s) of the body within about 10 minutes post injection, turnover rate of the substrate's metabolic pool can be calculated in the absence of significant changes in pool size during the mixing and measuring period.

Of further importance is the fact that the percentage of injected substrate converted to  $\text{CO}_2$  can be predicted with good accuracy from the rate constant,  $k$ . Figure 4 shows the regression line of slope versus percentage of injected label expired for periods of 2-3 hours post injection. The points represent determinations with acetate-1- $^{14}\text{C}$  injected into rats whose age and weight varied from 90-240 days and 200-500 grams,

respectively. The correlation coefficient of 0.958 indicates that the slope, determined during the first hour post injection, can serve to predict the conversion of labeled acetate for up to three hours. It is likely that little loss of accuracy would occur for longer collection periods since a substantial amount of the injected compound is expired during the first hour after injection. With acetate-1-<sup>14</sup>C, we have consistently recovered 40-50% and as much as 75% of the injected label during the first 60 minutes after injection. These values agree with the data of Gould, et al (3) who recovered 50% of acetate-1-<sup>14</sup>C from rats in 42 minutes.

We have found the slope values to be repeatable within ± 5% when at least 4 animals are used. With groups of 6-12 animals, the sensitivity is such that changes in catabolic rate due to time of day, prandial state, and reaction to stress of handling are readily apparent. It is, however, necessary to maintain a minimum level of total activity injected into the animals. A group of 3 or 4 animals require 100-150  $\mu$ Ci/kg of body weight to insure sufficient expired activity. Groups of 6-8 rats were customarily injected with 50  $\mu$ Ci/kg while a 12-rat group needed only 25  $\mu$ Ci/kg for sufficient activity in the expired CO<sub>2</sub>. In the hamster experiments, it can be seen (Table I.) that neither the number of animals injected (2, 5 or 10) or the dose (25, 50 or 100  $\mu$ Ci/kg) had any substantial effect on either the slope constant or the total activity expired.

The statistical analyses in Table 2 indicate that there were significant correlations and regressions for both the slope constant k

Table I. Results of Various Treatment Combinations Involving Number of Animals Injected and Dose ( $\mu\text{Ci/kg}$ ) for  $\dot{V}\text{O}_2$  ( $\text{L/six hr kg}^{-1}$ ), Slope Constant  $k$  and Activity Expired as a Function of Activity Injected.

<u>Treatment</u>	<u>No. Animals Injected</u>	<u>Dose</u>	<u><math>\dot{V}\text{O}_2</math></u>	<u><math>k(\times 10^3)</math></u>	<u>dpm expired dpm injected</u>
Air	10	50	9.50	6.32	.460
Air	10	50	9.74	7.08	.563
Air	10	50	10.19	9.19	.581
Air	10	25	9.39	6.60	.471
Air	10	100	10.92	9.33	.604
Air	5	100	8.74	8.00	.550
Air	2	100	10.73	8.39	.579
Ar/ $\text{O}_2$	10	50	9.81	7.12	.482
$\text{N}_2\text{O}/\text{O}_2$	10	50	11.69	5.72	.488
Air ( $12^\circ\text{C}$ )	10	50	14.60	11.65	.637
Air (barotrauma)	10	50	8.70	5.15	.439
Air (contaminate w/Ar)	10	50	10.00	7.65	.480

Table II. Statistical Results of Comparison of k Values Versus  $\frac{\text{dpm expired}}{\text{dpm injected}}$  as an Indicator of Oxygen Consumption. (n = 12)

	b	s.d.	t	p	r	p
k	.786	1.419	2.94	.025	.690	.01
$\frac{\text{dpm expired}}{\text{dpm injected}}$	.0252	.0532	2.52	.050	.619	.05

and total expired activity as a function of oxygen consumption. However, the lower probability levels of the k data for both correlation and regression analyses suggest that the mathematical treatment offers a more precise and reliable estimate of oxygen consumption, and therefore metabolic rate, than does accumulative expired activity.

## Discussion

Requisite with the use of this analysis as an indicator of metabolic rate are three assumptions. First, changes in the pool size must be known or assumed to not occur under the experimental conditions. Without plasma acetate specific activity measurements, the size of the pool diluted by the tracer cannot be known; if pool size does change, then an alteration in metabolic rate might be suggested when none, in fact, occurred.

Second, changes in the fraction of the total acetate pool entering the TCA cycle must be known or assumed to not occur. If changes in the amount of label entering the TCA cycle do occur, an alteration in metabolic rate would be implied when none had occurred. Such a situation is readily detected by measuring  $\text{CO}_2$  specific activity. Since lipid biosynthesis is the only major alternative for acetate utilization, changes in the rate of lipid synthesis can be measured by incorporation studies. In the present circumstances, we have not found appreciable changes in the fraction of total acetate entering the TCA cycle and have, thus, been able to dispense with expired  $\text{CO}_2$  specific activity measurements.

Third, it must be assumed that the appearance of  $^{14}\text{CO}_2$  in the expired gas is proportional to its actual rate of oxidation; the experimental conditions should not differentially alter those factors influencing the correlation between actual oxidation rate and appearance as  $\text{CO}_2$  in

the expired gas. These factors include  $\text{CO}_2$  diffusion constants, blood supply to the tissues, exchange of  $^{14}\text{CO}_2$  with total body  $\text{CO}_2$  "stores", and ventilation rates. Gould, et al (3) pointed out that the rate of catabolism and expiration of  $^{14}\text{CO}_2$  can vary with the substrate. They found the two rates did not differ in the case of acetate or bicarbonate but that succinate oxidation was faster than its appearance as  $\text{CO}_2$  in the expired gas.

A further consideration, although untested as yet, is that the pathway by which exogenous acetate enters the cell may be influenced by the experimental conditions while endogenous acetate is not. The latter enters an acetate pool primarily as a result of either carbohydrate or fatty acid catabolism, whereas injected acetate must cross the cell membrane. It is possible that the two routes of entry might be differentially affected by an experimental condition such that the injected substrate would not reflect the true endogenous situation. We feel that under our experimental conditions of mild physiological stress, neither the number of acetate pools which may exist nor the route of entry into the mitochondria complicate interpretation of the results.

The use of acetate as the test substrate minimizes these problems while offering an optimal indicator for aerobic oxidation. Since the TCA cycle is dependent on acetate as a substrate its activity must be sensitively reflected in acetate pool flux. An additional advantage is that acetate uptake by the cycle must be proportional to oxygen uptake by the cell and

CO<sub>2</sub> produced from it since the major amount of oxygen consumption and CO<sub>2</sub> production occurs via the TCA cycle. In our hands, the labeled substrate method is significantly more sensitive than the measurement of oxygen consumption or total CO<sub>2</sub> production.

Unlike many other metabolic substrates, acetate is used principally for fatty acid synthesis, steroid synthesis and TCA cycle oxidation. With inputs into the acetate pool mainly from pyruvate and fatty acid oxidation, and outflow into the TCA cycle and lipid synthesis pathways, the variables affecting pool size and turnover rate are limited. It is unlikely that minor pathways such as ketone metabolism significantly influence acetate pool characteristics in all but exceptional circumstances or specific tissues. Thus, if in a given experiment there is some doubt about the validity of the assumption that the fraction of the total acetate pool entering the TCA cycle has not occurred, excellent challenge data can be obtained by simply measuring the uptake of the acetate label into the total body lipid as a function of time.

We have used labeled glucose in a similar manner with good results. Since its pool fluctuates more drastically, correlation of its rate constant with metabolic rate indicated by other parameters has not been quite as good as with acetate. Using glucose-1-<sup>14</sup>C and glucose-6-<sup>14</sup>C and a modification of the mathematical procedure of Katz, Landau and Bartsch (5), we have been able to quantify both the aerobic and anaerobic catabolism of glucose in rats (2). Rats in a marginally hyperoxic environment were



found to have "normal" total  $\text{CO}_2$  production despite partial inhibition of the pyruvate and succinate dehydrogenase systems, which caused disruption of the TCA cycle as indicated by gas-solid chromatography of TCA cycle intermediates. It was found that in the hyperoxic animals, twice as much glucose was catabolized via the pentose phosphate pathway at the expense of the glycolytic pathway. This accounted for the normal total  $\text{CO}_2$  production despite a slowing of the TCA cycle.

In this same series of experiments, analysis of  $^{14}\text{CO}_2$  expiration from injected pyruvate-2- $^{14}\text{C}$  demonstrated conclusively that pyruvate conversion to acetyl-CoA was altered in the hyperoxic state. It should be emphasized that the method allows the animals to remain in as normal a state as is possible under the conditions. This is particularly important when subtle metabolic changes are being examined since starvation, restraint, repeated withdrawal of blood, or removal to a foreign testing apparatus often changes either the rate or the nature of metabolism so as to obscure the effects of the test variable. Apart from the initial injection, our rats were tested in the same environment and physiological state to which they had become accustomed; the fact that the slope constants predictably varied with the often marginal stressors to which the animals were exposed is consistent with the concept that the results are uncomplicated appreciably by other factors.

Of great importance, too, is the fact that the analytical procedure is quick and simple. We have adapted a computer key punch to a liquid

scintillation counter which allows the counts to be prepared directly for computer processing on the same day as the samples are counted.

Footnote

\*PPO =2,5-Diphenyloxazole, New England Nuclear Corporation

POPOP =1,4 bis (2 (4-methyl-5-phenyloxazolyl) )-Benzene,

New England Nuclear Corporation

Cab-O-Sil-thixotropic gel, Cabot Corporation

Figure 1. Graph of accumulative disintegrations per minute versus time in expired  $\text{CO}_2$  from 12 rats injected with sodium acetate-1- $^{14}\text{C}$ .

Figure 2. Graph of the logarithm of activity remaining in the body versus time and a least squares regression analysis of the data.

Figure 3. Graph showing regression analyses of data obtained from rats exposed to either  $\text{Ne-O}_2$  or air for four weeks.

Figure 4. Graph of rate constant "k" versus the percentage of injected label expired as  $^{14}\text{CO}_2$  at three hours past injection. The data were taken from 25 experiments in which acetate-1- $^{14}\text{C}$  or glucose-UL- $^{14}\text{C}$  were injected.

